

Gonadal Organization and Gametogenesis in the Fresh-water Mussel *Diplodon chilensis chilensis* (Mollusca: Bivalvia)

by

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Abstract. The gonadal organization and cytological characteristics of gametogenesis in the fresh-water mussel *Diplodon chilensis chilensis* are described. Although the sexes did not differ macroscopically, gonadal sections demonstrate the existence of gonochoric individuals in which testis and ovary are clearly distinct. In both sexes, gonads are ramified organs bearing numerous follicles closely packed among the intestine coils. Follicles in males contain gametes at different stages of maturation which can be recognized by their shape, size, and nuclear features. They are organized in clusters of spermatogonia, primary and secondary spermatocytes, and spermatids. Spermatozoa are accumulated in the lumen of follicles. Follicles of the female gonad contain oogonia and oocytes at different stages of development. Morphological features similar to those described in other fresh-water and marine bivalves are apparent. In the specimens of the size classes examined, no differences in the gonadal organization and gametogenesis were observed during the study period.

INTRODUCTION

DUE TO CHILE's extensive coastline (approximately 4200 km) and the subsequent diversity and abundance of marine invertebrates, most of the studies about reproduction and other aspects of the biology of mollusks have been carried out on marine species, especially those of commercial value. A review of the literature shows that many aspects of the biology of the fresh-water mollusks inhabiting Chile have never been studied. Practically nothing is known about the reproduction of the fresh-water mussel *Diplodon chilensis chilensis* (Gray, 1828) although it is abundant in rivers and lakes of Chile and ranges from Arica in the North (18°30') to the Strait of Magellan in the southern part of the country (53°). *Diplodon chilensis chilensis* is a hardy bivalve that tolerates temperature and oxygen level changes and possesses a high filtration capacity (BUSSE, 1970).

Due to the vulnerability of the gonadic tissue to environmental contaminants such as organochlorine compounds, pesticides, and heavy metals, the study of the reproduction of *Diplodon chilensis chilensis* is of importance in biological monitoring studies. This species may prove useful as an indicator of environmental changes due to fresh-water pollution, which in turn may be a source of pollution of coastal waters. The subsequent negative ef-

fects of such pollutants on littoral communities have been reported in several studies (KNAUER & MARTIN, 1972; WILLIAMS & WEISS, 1973; BERGE & HILLEBRAND, 1974; WOLF, 1975; REIJNDERS, 1980).

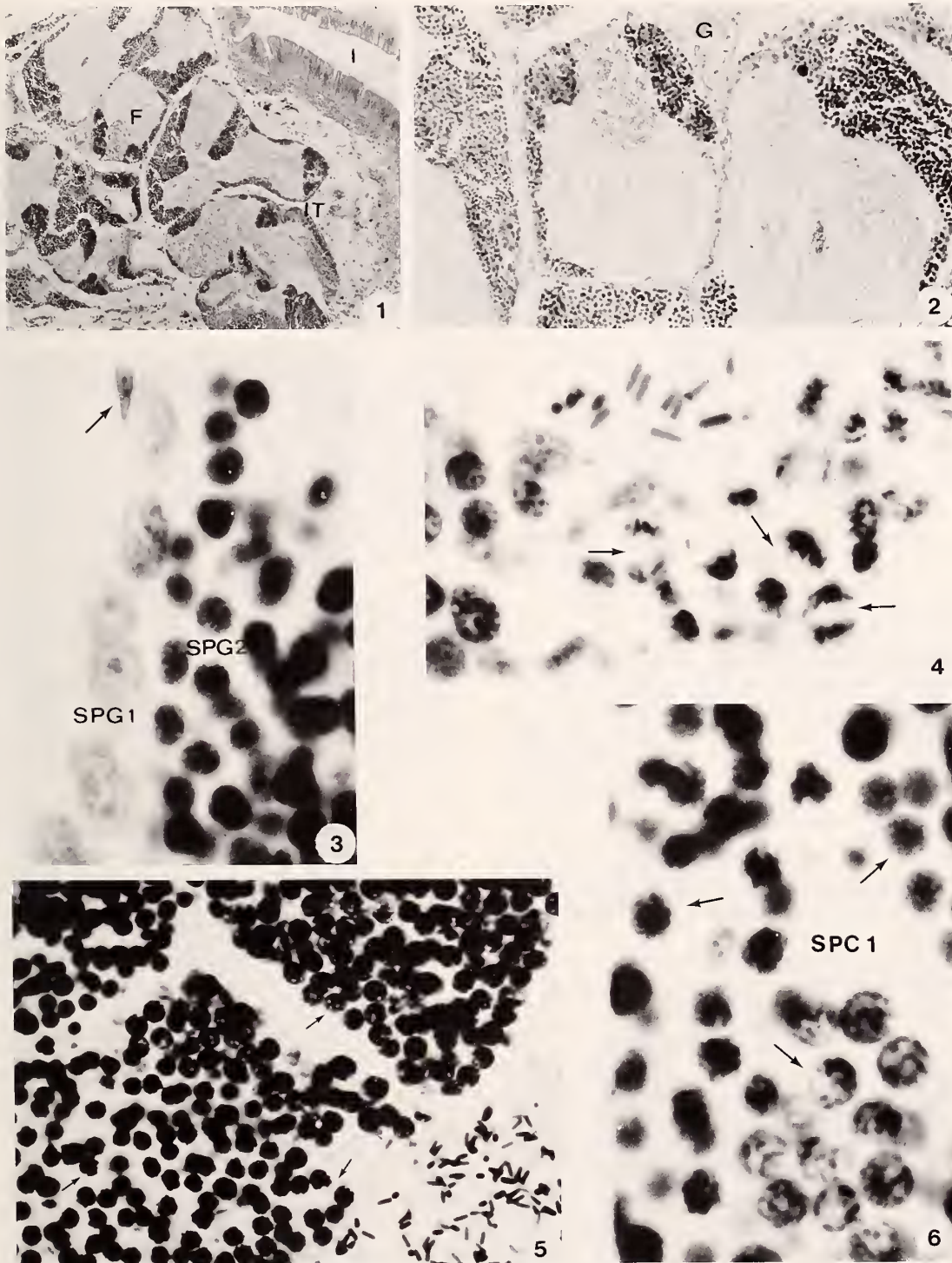
The present study analyzes the histology of the gonads and the cytological characteristics of gametogenesis in *Diplodon chilensis chilensis*.

MATERIALS AND METHODS

In March 1982, specimens of *Diplodon chilensis chilensis* were collected at random from shallow waters of Lake Villarrica (39°17'S, 72°13'W). The mussels lived at depths ranging from 20 to 60 cm. The individuals were distributed in eight sizes grouped in the following classes: S₁ (16-25 mm); S₂ (26-30 mm); S₃ (31-35 mm); S₄ (36-40 mm); S₅ (41-45 mm); S₆ (46-50 mm); S₇ (51-55 mm); and S₈ (56-65 mm).

Five individuals were selected at random from each size class. The sex was determined by gonad smears. The viscera were fixed in aqueous Bouin's fixative. After embedding in paraffin, serial sections were cut at 7 µm and stained with hematoxylin and eosin.

The gonadal organization and the cytological characteristics of the germinal and somatic cells of both sexes were inspected using a light microscope.



Explanation of Figures 1 to 6

Figure 1. A topographical view of the male gonad of *Diplodon chilensis chilensis*. The well delimited follicles (F) occupy the visceral mass (mesosoma) surrounding the intestine (I). Scanty interstitial tissue (IT) is present among follicles. $\times 20$.

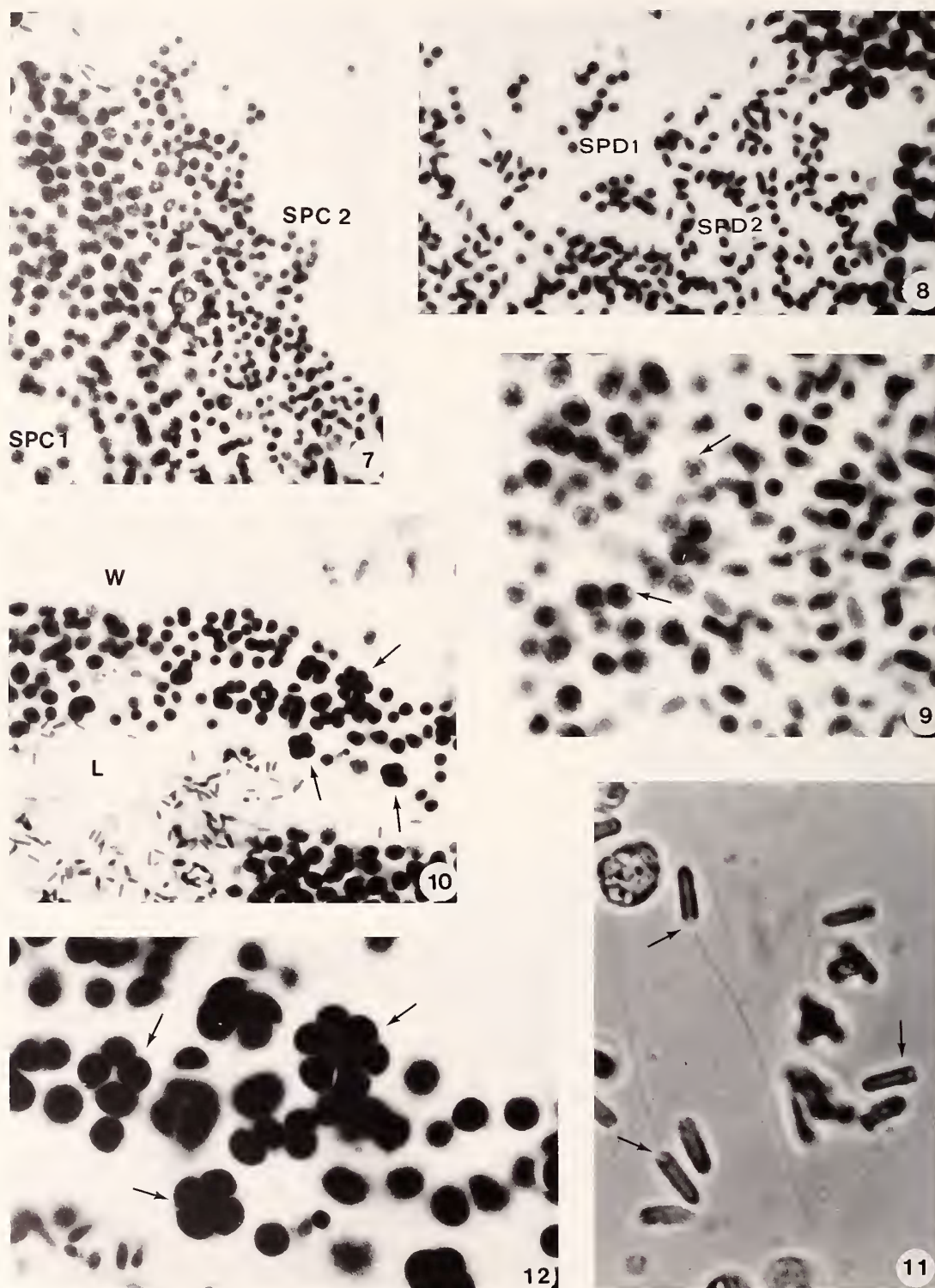
Figure 2. Cell clusters within follicles. A gonoduct (G) is seen next to the follicles. $\times 50$.

Figure 3. Type 1 spermatogonia (SPG 1) in single rows and

type 2 spermatogonia (SPG 2) in clusters in a follicle. The spindle-shaped nucleus (arrow) is from a cell of the follicle wall. $\times 500$.

Figure 4. Type 1 spermatogonia showing some mitotic figures (arrows). $\times 500$.

Figures 5, 6. Clusters of primary spermatocytes (SPC 1) showing meiotic prophase configurations (arrows). $\times 200$, $\times 500$.



Explanation of Figures 7 to 12

Figure 7. Clusters of secondary spermatocytes (SPC 2) interspersed with spermatids with rounded nuclei. The nuclei of these cell types are hardly distinguishable. Next to them are primary spermatocyte nuclei (SPC 1). $\times 200$.

Figures 8, 9. Clusters of spermatids with rounded nuclei (SPD 1) and elongate nuclei (SPD 2). The acrosomal vacuole can be seen (arrows). $\times 200$, $\times 500$.

RESULTS

Diplodon chilensis chilensis is dioecious, as are the great majority of bivalves, and sexual dimorphism is absent.

Male Gonad and Germ Cells

The male gonad consists of numerous follicles located in the visceral mass surrounding the intestinal coils. The follicles show diversity in shape and size and are delimited by a thin cellular enveloping membrane (Ancel's layer) which may have some connective fibers applied to it. Scanty interstitial (connective) tissue surrounds the follicles (Figure 1).

The follicles are crowded with cells at different stages of spermatogenesis. The cells of particular stages can be recognized by their shape, size, and nuclear features, and are arranged in groups or clusters markedly delimited and located at the periphery of the follicles (Figure 2).

The gonoducts are branched and smaller in diameter than follicles; a lumen is always present. The walls are lined with a single row of ciliated columnar cells with elliptical nuclei. The cytoplasm stains lightly with eosin (Figure 2).

Spermatogonia: Two types of spermatogonia can be recognized. Those of the first type are less numerous than the second and lie in a single row against the membrane enveloping the follicles. These spermatogonia have large vesicular nuclei (9.0–10 μm) with a conspicuous nucleolus and loose chromatin (Figure 3). Fibroblast-like cells are seen next to this type of spermatogonia. The fibroblast-like cells are in the follicle wall. Their nuclei are spindle shaped, and the cytoplasm is difficult to visualize (Figure 3). The second type of spermatogonia occurs in clusters without visible cytoplasm and lies close to, or sometimes interspersed with, primary spermatocytes. The densely reticulate nuclei possess no nucleoli and are smaller and stain more heavily than the first type of spermatogonia (Figure 3). Occasionally, mitotic figures can be seen in the first type of spermatogonia (Figure 4).

Primary spermatocytes: These cells form numerous and compact clusters. They have a fine, faintly staining plasma membrane and a clear, scanty cytoplasm, but these features are barely visible in the congested mass of nuclei. The nuclei vary in appearance as the chromatin assumes different consistencies and locations within the nucleus. The chromosomes can be scattered in the nucleus or they may be polarized at the periphery showing typical figures of meiotic prophase (Figures 5, 6).

Secondary spermatocytes: Secondary spermatocytes are seen less commonly than primary spermatocytes. They

occur in groups generally intermingled with spermatids forming mixed cell groups (Figure 7). Secondary spermatocytes have small, round nuclei (3.0 μm) with granular and heavily staining chromatin.

Spermatids: The nucleus of one type of spermatid is similar in shape, size, and staining properties to that of the secondary spermatocytes (Figure 8). A second type of nucleus can be seen in the spermatid groups. These nuclei are elongate, stain heavily and homogeneously, and have a short eosinophilic zone at one end (Figures 8, 9). They are intermingled with the nuclei of the first type of spermatid which show a vacuole similar to the acrosomal granule (Figure 9).

Spermatozoa: Spermatozoa are formed in the center of the follicles where they accumulate. The mature spermatozoon has an elongate head about 4.0 μm long and 1.5 μm wide. The chromatin is dense and stains homogeneously (Figure 10). Fresh smears of gonad tissue show flagella four to five times the length of the heads (20–24 μm) emerging from the eosinophilic mass at one end of the sperm heads (Figure 11).

In addition to normal germinal cells, the follicles of many males also contain numerous multinucleated spherical dark bodies (Figure 12) similar to those described in *Mya arenaria* (COE & TURNER, 1938; SHAW, 1965; PORTER, 1974) and *Cyprina islandica* (LOOSANOFF, 1953). Such dark bodies are usually closer to the periphery of the follicles than to their center, and contain a variable number of pycnotic nuclei (from 2 to 10 nuclei); alternatively, they can be seen as single nuclei (Figure 12).

Female Gonad and Germ Cells

As in males, the female gonad of *Diplodon chilensis chilensis* is a branched gland embedded in the visceral mass. Numerous follicles surround the coils of the intestine (Figure 13). The follicles are irregular in size and shape, and are limited by a connective tissue wall of variable thickness.

In the follicles, germ cells at different stages of development can be recognized by their size, shape, and staining properties (Figure 13).

Oogonia: Oogonia are observed embedded in the follicle walls. They are distinguished from the connective tissue cells by their relatively large size and highly chromatic nuclei (Figures 14, 15).

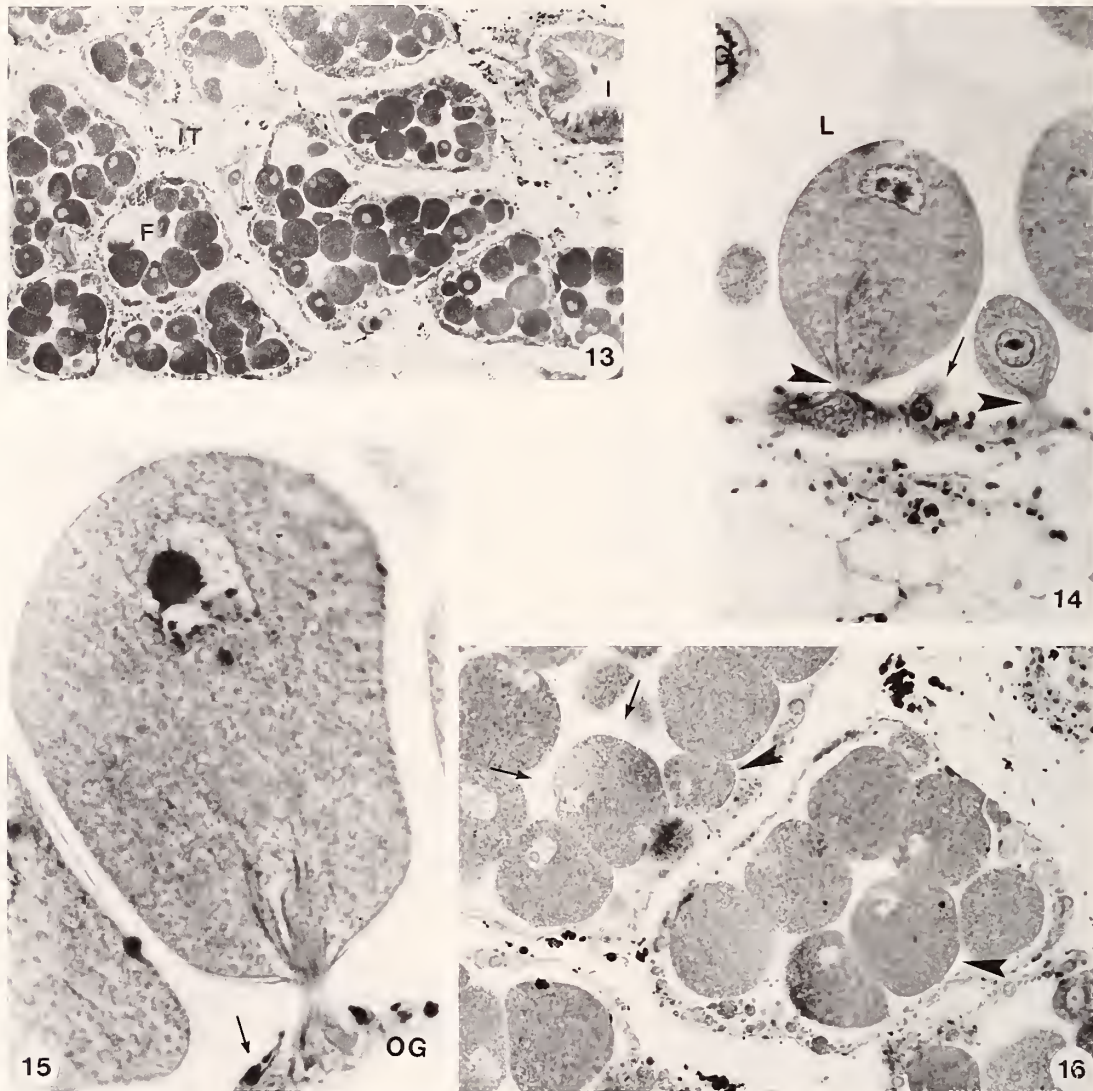
Growing oocytes—previtellogenic oocytes: The shape of the newly formed oocytes may be square, cylindrical, or hemispherical (Figures 13, 14). They bulge from the

Figure 10. Sperm in the lumen of a follicle (L). Next to the wall (W) lie multinucleated spherical bodies (arrows) of atypical spermatogenesis. $\times 200$.

Figure 11. Gonad smear with spermatozoa. The flagellum can

be seen emerging from the eosinophilic end of the sperm head (midpiece) (arrows). $\times 500$.

Figure 12. Multinucleated spherical bodies (atypical spermatogenesis). $\times 500$.



Explanation of Figures 13 to 16

Figure 13. Topographical view of the female gonad of *Diplodon chilensis chilensis*. The follicles (F) in the visceral mass surround the intestine (I). Scanty interstitial tissue (IT) is present among follicles. $\times 20$.

Figure 14. Previtellogenic oocyte bulging from the follicle wall (arrow). Protruding into the lumen (L) growing oocytes can be seen attached by a cytoplasmic stalk (arrow head). $\times 500$.

follicle wall, and the largest ones are attached to the follicle wall by a short, broad stalk (Figure 14). The cytoplasm is basophilic and the nucleus is large, stains lightly, and has disperse chromatin with one or two prominent basophilic or eosinophilic nucleoli (Figure 14).

Growing oocytes—vitellogenic oocytes: As the oocytes grow they elongate and protrude into the center of the follicles. The nucleus migrates to the distal or free end of the cell (Figures 14, 15). The stalk becomes thinner and

Figure 15. Growing (vitellogenic) oocyte with slender stalk. Oogonium (OG) and wall cell nuclei (arrow) embedded in the follicle wall. $\times 500$.

Figure 16. Full grown oocytes in follicles. The vitelline membrane is sloughed away in some (arrows). Growing oocytes (arrow heads). A gonoduct can be seen. $\times 500$.

the oocytes increase in size as yolk accumulates. Bundles of fibrillar material can be seen in the stalk and in the cytoplasm close to the stalk (Figure 15). The nucleus enlarges and stains less heavily as the chromatin disperses. The nucleoli continue to grow and in some oocytes are seen to be eosinophilic or basophilic.

Full grown oocytes (morphologically mature): These oocytes have become freed from the follicle wall and have moved into the lumen. They are more regular in outline

than the attached oocytes, and they are larger than vitellogenic oocytes, even though some of the latter can reach a larger size than the free ones. Full grown oocytes can reach up to 150 μm in diameter. The germinal vesicle is intact and lightly stained, with dispersed chromatin; the nucleoli are prominent. The cytoplasm is loaded with vitelline platelets (Figure 16).

One can see in developed oocytes (growing and full grown oocytes) a covering, the vitelline membrane, which is rather thick and prominent close to the cytoplasmic stalk in attached oocytes (Figures 15, 16).

The interstitial tissue consists of vesicular connective tissue in which are abundant cells similar to those called Cell Type A by TRANTER (1958) although they were not seen within the follicles in the present study. These cells are especially abundant in the tissue surrounding the intestine (Figure 13).

The gonoducts show the same features described for the gonoducts of the male gonad (Figures 14, 16).

DISCUSSION

The structure of the gonad of *Diplodon chilensis chilensis* corresponds largely to that described for the fresh-water mussels *Anodonta cygnea* (PURCHON, 1968), *Sphaerium simile* (GILMORE, 1917), *Lamellidens corrianus* (NAGABHUSHANAM & LOHGAONKER, 1978), and some marine bivalves, in which the gonad is a branched gland that terminates in a network of follicles occupying a large part of the visceral mass. A system of branched ducts, the gonoducts, evacuate ripe germ cells during spawning periods. *Diplodon ch. chilensis* differs from other bivalves, such as *Mytilus edulis*, in which the gonad penetrates into mantle tissue (SASTRY, 1977). Copulatory organs and accessory glands are absent.

Although macroscopic sexual dimorphism is not present, microscopic observations show that *Diplodon ch. chilensis* is strictly gonochoric, because even in the smallest specimens sampled (16 mm) only male or female follicles could be recognized in the same individual.

Male germ cells correspond with the usual types formed during spermatogenesis. No indifferent or primordial germ cells, described for some pelecypods (TRANTER, 1958; LUBET, 1959), were recognized in *Diplodon ch. chilensis*. At least two types of spermatogonia can be recognized. The first type (type 1 spermatogonia) would be primitive spermatogonia, less advanced than the second type recognized (type 2 spermatogonia) which would be definitive spermatogonia since these are the end products of spermatogonial mitosis. Type 2 spermatogonia directly give rise to primary spermatocytes.

The variable appearance of the nuclei of primary spermatocytes corresponds to the various configurations of meiotic prophase (Figures 5, 6). Primary spermatocytes give rise to secondary spermatocytes. These are seen less commonly than their predecessors. Apparently, division is very rapid at this stage, with the secondary spermatocytes

giving rise to the first type of spermatid characterized by the nuclear vacuole, which would correspond to the onset of the acrosomal granule formation. Spermatids with elongated nuclei are cells in advanced stages of differentiation into spermatozoa.

The eosinophilic zone described in one of the ends of the sperm head corresponds to the midpiece of the spermatozoa and possibly consists of an aggregate of mitochondria as described in *Haliotis rufescens* (YOUNG & DE MARTINI, 1970). This view is supported by the examination of fresh smears in which the flagellum is seen emerging from the eosinophilic mass (Figure 11).

The multinucleated spherical dark bodies observed in the follicles of many males (Figures 10, 12) would be cells of atypical spermatogenesis according to LOOSANOFF (1953) who described them in the spermatogenesis of *Cyprina islandica* and earlier in *Mercenaria mercenaria* (LOOSANOFF, 1937a). They have also been described in *Mya arenaria* (COE & TURNER, 1938; SHAW, 1965; PORTER, 1974) as well as in the unionids *Anodonta anatina* (BLOOMER, 1936), *A. cygnea* (BLOOMER, 1946), and *A. grandis* (VAN DER SCHALIE & LOCKE, 1941). According to HEARD (1975) these abnormal cells are of widespread occurrence in the Unionacea, having been found in 43 species of 17 genera in the families Amblemidae, Hyriidae, Margaritiferidae, and Unionidae. COE & TURNER (1938) point out that in some cases such multinucleated dark bodies may break apart and continue further development, finally reaching the stage of spermatozoa. BLOOMER (1946) inferred that in *A. cygnea* these atypical cells (sperm-morulae) metamorphosed into mature spermatozoa based on the observed disappearance of sperm morulae just prior to the appearance of large numbers of spermatozoa. More often they become pycnotic and are cytolized. The observations made in the present study do not elucidate the ultimate fate of these abnormal cells.

Oogenesis in *Diplodon ch. chilensis* has the usual characteristics described for other fresh-water and marine mollusks. Growing oocytes with a cytoplasmic stalk have also been described for *Anodonta* (BEAMS & SEKHON, 1966), *Sphaerium simile* (ZUMOFF, 1973), and in several Chilean marine bivalves (CIFUENTES, 1975; LOZADA & REYES, 1981). In gastropods, HUAQUÍN (1979) described previtellogenic oocytes attached by a stalk to the germinative epithelium of the ovary in *Concholepas concholepas*, and a similar situation was described for *Haliotis rufescens* (YOUNG & DE MARTINI, 1970). BEAMS & SEKHON (1966) described abundant and closely packed microtubules in the stalks of developing oocytes. These authors assign a mechanical and also a possible nutritional role for these microtubules in the growing oocyte.

The observations made in the above-mentioned several species and those made in the present study indicate that oocytes apparently remain attached by means of the cytoplasmic stalk to the ovarian wall during much of their development. In *Diplodon ch. chilensis* oocytes up to 120 μm in diameter could be seen to be connected to the fol-

lucular wall, although it was not possible to determine exactly when they became detached.

An intact germinal vesicle in full grown oocytes indicates that they are not physiologically mature. In these oocytes meiosis either may be arrested at early prophase, in the vegetative phase (RAVEN, 1966), or meiosis may not have started yet, as has been reported for other bivalves (LOOSANOFF, 1937a, b; 1953). Therefore, in *Diplodon ch. chilensis* either the germinal vesicle would initiate maturation just before spawning, or eggs are released with the nucleus intact and the initiation of meiosis would occur after the sperm has penetrated the egg (activation), as has been reported for other fresh-water bivalves (OKADA, 1935; ZUMOFF, 1973) and marine mollusks (BRETSCHNEIDER & RAVEN, 1951; GALSTOFF, 1961; STICKNEY, 1963). LILLIE (1901) reported that in the unionid *Elliptio complanatus* diploid primary oocytes and not mature haploid ova were released from the ovaries and the reduction division of these cells did not occur until after sperm penetration (in the marsupial demibranchs).

The rather thick vitelline membrane that covers oocytes of *Diplodon ch. chilensis* is characteristic of many fresh-water bivalves (FRETTER & GRAHAM, 1964). No evidence of a second cover, the chorion, as described for several bivalves including the fresh-water mussel *Anodonta* (BEAMS & SEKHON, 1966), have been found in *Diplodon ch. chilensis*.

The meaning and significance of interstitial cells seen in males and females of *Diplodon ch. chilensis* is not clear. LOOSANOFF (1937a, b) assigns to cells of the surrounding connective tissue (amoebocytes) a nutritional role in the active phase of the reproductive cycle of *Mercenaria mercenaria*. TRANTER (1958) ascribes to these cells a resorptive function in follicles emptied from gametes after spawning and during early development in *Pinctada albina*. No evidence for any of these roles could be obtained in the present study since interstitial cells were always seen outside the follicles and with no connection to the germinal cells.

There was no observed difference in gametogenesis in different size classes. *Diplodon ch. chilensis* reaches sexual maturity at a size smaller than 16 mm, the size of the smallest specimens analyzed in this study.

ACKNOWLEDGMENTS

This work was supported with funds from the Dirección de Investigación, Catholic University of Chile-Santiago and Comisión de Investigación, Catholic University of Chile-Temuco. The authors are indebted to Dr. E. Bustos-Obregón for his valuable suggestions and to V. Leyton and S. Bustos for their technical assistance and critical reading of the manuscript.

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